The CXCR3 Activating Chemokines IP-10, Mig, and IP-9 are Expressed in Allergic but not in Irritant Patch Test Reactions

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Differentiation between allergic and irritant contact dermatitis reactions is difficult, as both inflammatory diseases are clinically, histologically, and immunohistologically very similar. Previous studies in mice revealed that the chemokine IP-10 is exclusively expressed in allergic contact dermatitis reactions. In the present study, we investigated whether the mRNA expression of IP-10 and the related CXCR3 activating chemokines, Mig and IP-9 are also differentially expressed in human allergic contact dermatitis and irritant contact dermatitis reactions. Skin biopsies from allergic (13 cases) and sodium lauryl sulfate-induced irritant patch test reactions (13 cases), obtained 1–72 h after patch testing, were studied by means of an *in situ* hybridization technique. Results of chemokine mRNA expression were correlated with clinical scoring, histology, and immunohistochemical data including the proportion of inflammatory cells expressing CXCR3, the receptor for IP-10, Mig, and IP-9, and ICAM-1 and HLA-DR expression on keratinocytes. IP-10, Mig, and IP-9 mRNA were detected in seven of nine allergic contact dermatitis reactions after 24–72 h, but not in sodium lauryl sulfate-induced irritant contact dermatitis reactions. ICAM-1 expression by keratinocytes was only found in allergic contact dermatitis reactions and correlated with chemokine expression. Moreover, up to 50% of the infiltrating cells in allergic contact dermatitis expressed CXCR3, in contrast to only 20% in irritant contact dermatitis reactions. In conclusion, we have demonstrated differences in chemokine expression between allergic contact dermatitis and irritant contact dermatitis reactions, which might reflect different regulatory mechanisms operating in these diseases and may be an important clue for differentiation between allergic contact dermatitis and irritant contact dermatitis reactions. *Key words: allergic contact dermatitis/***in situ** *hybridization/irritant contact dermatitis. J Invest Dermatol 113:574–578, 1999*

Differentiation between allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) is difficult, both clinically and histologically. For this reason, in the last two decades many studies have attempted to define immunophenotypical criteria, allowing differentiation between both conditions. The results of these studies, however, have revealed similarities rather than clear-cut differences between ACD and ICD, despite the different pathogenic mechanisms involved in the development of these conditions. These similarities concerned both the localization and cellular composition of the skin infiltrates (Scheynius *et al*, 1984; Willis *et al*, 1986), the expression of activation markers expressed by keratinocytes, such as ICAM-1 and HLA-DR (Gawkrodger *et al*, 1987; Brasch *et al*, 1992) and cytokine profiles (Hoefakker *et al*, 1995). Taken together, these studies have been unable to define useful histologic or immunohistologic criteria allowing differentiation between both conditions.

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Abbreviations: ACD, allergic contact dermatitis; ICD, irritant contact dermatitis; IP-10, interferon-γ-induced protein 10; IP-9, interferon-γ-induced protein 9; Mig, monokine induced by interferon-γ.

In contrast to these studies in humans, studies in mice revealed different cytokine expression patterns between ACD and ICD. Using a reverse transcriptase–polymerase chain reaction technique, Enk and Katz (1992) found expression of interleukin-1 and IP-10 mRNA in ACD but not in ICD induced by sodium lauryl sulfate (SLS). IP-10 (Luster *et al*, 1985) is a member of the CXC family of chemokines and specifically attracts activated T cells expressing the chemokine receptor CXCR3 (Loetscher *et al*, 1996; Qin *et al*, 1998). Moreover, IP-10 has been shown to be produced abundantly by keratinocytes upon stimulation by interferon (IFN) -γ both *in vitro* (Boorsma *et al*, 1994, 1998) and *in vivo* (Kaplan *et al*, 1987; Tensen *et al*, 1998). Two other members of the CXC family that act through the same receptor are Mig (Farber, 1993) and IP-9 (Tensen *et al*, 1999a). IP-9 is a novel highly potent ligand for CXCR3, recently isolated from IFN-γ stimulated keratinocytes in its native biologically active form (Tensen *et al*, 1999a). The cDNA encoding IP-9 is also known as b-R1 (Rani *et al*, 1996), H174 (Jacobs *et al*, 1997) or I-TAC (Cole *et al*, 1998).

The exclusive expression of IP-10 in ACD in mice, as observed by Enk and Katz (1992), prompted us to study mRNA expression of IP-10, and the closely related chemokines Mig and IP-9, in human allergic and irritant patch test reactions using *in situ* hybridization. As IP-10, Mig, and IP-9 are induced by IFN-γ the expression of these chemokines was correlated with the expression of ICAM-1 and HLA-DR, which are induced by IFN-γ as well (Griffiths *et al*, 1989). Furthermore, the presence of CXCR3,

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Table I. Allergic test subjects, patch tests, and schedule for taking biopsies

Case no.	Allergen	biopsy (h)
1	Coumarin	4 and 24
2	Nickel sulfate	4 and 24
3	Neomycin	4 and 24
4	Balsam of Peru	4 and 24
5	Kathon CG	48
6	Kathon CG	72
7	Methyldibromoglutaronitril	72
8	Imidazolidinyl urea	72
9	Epoxy resin	72
10	Sesquiterpene lactone mix	72
11	Carbamix	72
12	Epoxy resin	72
13	Thiuram	72

the receptor for IP-10, Mig, and IP-9, on infiltrating cells was investigated.

MATERIALS AND METHODS

Subjects Thirteen volunteers with a known contact allergy, were recruited from the contact dermatitis clinic. A further 13 healthy volunteers without contact allergy or dermatitis, were recruited from the university student population. The study was approved by the Hospital Ethical Committee and the volunteers participated after giving informed consent.

Allergic patch testing Contact allergy for various allergens (**Table I**) had originally been diagnosed by means of epicutaneous patch testing. For the study, these volunteers were patch tested on the back with allergens in generally accepted test concentrations with Van der Bend Square chambers (Bruynzeel *et al*, 1995). In the first group of four volunteers, the patches were removed and biopsies were taken after 4 and 24 h, respectively. In the second group of nine volunteers, the patches were removed after 48 h and biopsies were taken after 72 h. The patch test reactions were scored according to the International Contact Dermatitis Research Group (ICDRG) guidelines: – = negative reaction; + = weak positive reaction: redness, induration, and possibly papules; + + = positive reaction: erythema, induration, papules, and vesicles; + + + = strong positive reaction: intense erythema, induration, and coalescing vesicles.

Irritant (SLS) patch testing Thirteen healthy volunteers were patch tested with 5% SLS in distilled water. Fifty microliters were pipetted on filter-paper discs in Van der Bend Square chambers. The patches were applied to the lower back at three different spots. In five volunteers, patches were removed after 1, 3, and 6 h, respectively, and the biopsies were taken directly after the patches were removed. In another eight healthy volunteers, patches were removed after 24 h and a biopsy was taken at that time-point. In five of them, two more biopsies were taken at 48 h and at 72 h. Control biopsies were obtained from the vehicle patch tests. The patch test reactions were scored according to the ICDRG guidelines. The biopsies were embedded in Cryomatrix (Shandon, U.K.) and immediately frozen in liquid nitrogen.

Immunohistochemical analysis Five micrometer frozen tissue sections were fixed in acetone for 10 min at room temperature, and subsequently incubated with appropriately diluted primary antibody, biotinylated goat anti-mouse immunoglobulin antibody (DAKO, Denmark) and horseradish peroxidase-labeled streptavidin (DAKO). Horseradish peroxidase–enzyme activity was revealed using AEC/H_2O_2 as substrate solution, and the sections were counterstained with Mayers hematoxylin (Beljaards *et al*, 1997). The following primary antibodies were used: CD3 and CD4 (clones BB11 and BF5, respectively, Biosource International; Camarillo, CA), CD8 (clone BC8, Dr J. Wijdenes; Besançon, France), CD54 (clone MEM111, Monosan Uden, the Netherlands), HLA-DR (clone H2–5-10, Dutch Red Cross Central Laboratory of Blood Transfusion; Amsterdam, the Netherlands), neutrophil elastase (clone NP57, DAKO; Copenhagen, Denmark), and CXCR3 (clone 49801.111, R&D Systems, Minneapolis, MN).

Histologic examinations The amount of inflammatory cells, including CD3, CD4, CD8, and elastase positive cells, was assessed, using a three-point scale; –, few positive cells/as in normal skin; +, moderate presence of positive cells; ++, pronounced presence of positive cells. The dermal and epidermal compartments were examined separately.

***In situ* hybridization** Preparation of ^{35}S labeled RNA probes: pZERO plasmid DNA harboring IP-10 or Mig cDNA sequences (Tensen *et al*, 1998) or pTopo containing IP-9 cDNA (Tensen *et al*, 1999a) in either orientation were linearized and ^{35}S labeled sense (control) and anti-sense RNA probes were obtained by *in vitro* transcription with T7 RNA polymerase. After 2 h, the DNA templates were degraded with DNaseI and the labeled riboprobes were recovered by ethanol precipitation using 20 μg t-RNA as carrier, and subsequently dissolved in formamide. *In situ* hybridization procedure was performed as previously described (Tensen *et al*, 1998). After fixation of the cryostat sections, the slides were overlaid with 100 μl hybridization buffer containing 4×10^6 c.p.m. probe per 100 μl. Sections were mounted with coverslips and hybridized for 16–18 h at 50°C in an humidified chamber. After hybridization, nonhybridized probe was removed using high-stringency washes at 45°C. For autoradiography detection, slides were dipped in Ilford's K5 solution, exposed for 1–5 wk, then developed using Kodak D19, and counterstained with hematoxylin.

RESULTS

Clinical and histologic evaluation The allergic patch test reactions were all negative (-) after 4 h and weakly positive (+) after 24 h. After 48–72 h, two of nine were weakly positive (+) and seven of nine were positive (++) (**Table II**). The irritant patch test reactions were negative (-) after 1–6 h. After 24 h, five of eight were weakly positive (+) and three of eight were positive (++). After 48–72 h the irritant reactions were all weakly positive (+) (**Table II**). No clinical reactions were observed for the vehicle.

The density of the infiltrates paralleled the clinical scoring. Up to 4 h, the perivascular infiltrates in ACD resembled those in normal skin. From 24 to 72 h, the infiltrates increased from moderate to pronounced dense perivascular infiltrates with influx of some CD3 positive cells in the epidermis (**Table II**). A relatively constant percentage of CD3-positive cells (75%–90%) was observed, with a CD4/CD8 ratio of approximately 2:1. The infiltrates in ICD reactions were most pronounced at 24 h and contained a relatively high percentage of neutrophils (5%–25%) compared with less than 5% in ACD reactions. Superficial necrosis was also seen in a few cases of ICD reactions.

The percentage of inflammatory cells positive for the receptor CXCR3 in the ACD reactions varied between 25% and 50%, whereas in the ICD reactions 10%–30% of the cells were positive (**Table II**). The CXCR3 positive cells were observed mainly in the dermal infiltrates.

Expression of IP-10, Mig, and IP-9 mRNA The ACD reactions showed a time-dependent expression of IP-10, Mig, and IP-9 mRNA which concurred with the clinical manifestations. At 4 h no detectable signals were observed. At 24 h, few IP-10- and Mig-positive cells were seen at sites of close contact between inflammatory cells and the keratinocytes (two of four cases), whereas IP-9 mRNA was not detectable. After 48–72 h, IP-10, Mig, and IP-9 were positive in most of the biopsies. In seven of nine biopsies, IP-10 was found to be strongly expressed in the epidermis as well as the upper dermis (**Fig 1*a*, *b***; **Table II**). In the other two biopsies, IP-10 expression was minimal or absent. Consistently, in one of these cases, the clinical reaction was also weakly positive and the infiltrate was only moderate. The expression of Mig mRNA was similar to IP-10 mRNA expression, although, Mig was found more abundantly in the dermis (**Fig 1*c*, *d***; **Table II**). IP-9 mRNA was restricted to the epidermis and roughly paralleled IP-10 mRNA expression, but was generally less pronounced (**Fig 1*e*, *f***; **Table II**). In general, expression of chemokine mRNA correlated with the clinical reaction and the density of the infiltrates. In particular, where inflammatory cells were in close apposition to the epidermis, the keratinocytes as well as infiltrating cells abundantly expressed mRNA encoding chemokines.

Hybridization of the irritant patch test reactions with IP-10 (**Fig 2*a*, *b***), Mig, and IP-9 did not show any detectable signal up to 72 h. (**Table II**). Similarly, there was no detectable expression

Table II. Clinical, immunohistologic and *in situ* hybridization data from the allergic and irritant patch test reactions

	Allergic patch test reactions			Irritant patch test reactions		
n = number of biopsies	4 h n = 4	24 h n = 4	48–72 h n = 9	1–6 h n = 15	24 h n = 8	48–72 h n = 10
Clinical score[a]	–	+	+/++	–	+/++	+
Inflammatory cells[b]						
Epidermis	–	+[c]	+/++[c]	–	+/++[d]	+[d]
Dermis	–	+[c]	+/++[c]	–	+/++[d]	+/++[d]
Keratinocyte express[e]						
ICAM-1	–	±	+/++	±	±	±
HLA-DR	–	–	±/+	–	–	–
IP-10 expression[f]						
Epidermis	–	–/±	+/++	–	–	–
Dermis	–	–	+/++	–	–	–
Mig expression[f]						
Epidermis	–	–/±	+/++	–	–	–
Dermis	–	–	+/++	–	–	–
IP-9 expression[f]						
Epidermis	–	–	±/+	–	–	–
Dermis	–	–	±	–	–	–
CXCR3[g]		25–50%	25–50%		10–30%	10–30%

[a]–, negative reaction; +, weakly positive reaction; ++, positive reaction.
[b]–, few cells/as in normal skin; +, moderate infiltrate; ++, pronounced infiltrate.
[c]Mainly lymphocytes.
[d]Mixed infiltrates of mononuclear cells and neutrophils.
[e]–, no staining; ±, faint staining of basal layer keratinocytes; +, focal areas of strong staining of basal layer keratinocytes; ++, extensive areas of strong staining of basal and supra basal layer keratinocytes.
[f]–, no expression; ±, minimal expression; +, focal strong expression; ++, diffuse strong expression.
[g]Percentage of inflammatory cells expressing CXCR3.

of IP-10, Mig, or IP-9 mRNA in normal or control skin (data not shown).

ICAM-1 and HLA-DR expression by keratinocytes In the ACD reactions, the ICAM-1 expression by keratinocytes was only found at sites where inflammatory cells were in close contact with the epidermis, and correlated well with chemokine mRNA expression. At 4 h, the keratinocytes were consistently negative as in normal skin. From 24 to 72 h, the ICAM-1 expression increased from focal expression on basal layer keratinocytes to areas of extensive expression on basal and suprabasal layer keratinocytes (**Table II**). On the other hand, HLA-DR expression was not observed until 72 h, by then, only sparse foci of positive keratinocytes were observed (**Table II**). HLA-DR expression on keratinocytes did not correspond with the chemokine mRNA expression. In contrast with the ACD reactions, the ICD reactions were negative or showed weak focal expression for ICAM-1 from 1 h up to 72 h. In addition, HLA-DR expression was not detected in any of the ICD biopsies (**Table II**).

Besides keratinocytes, ICAM-1 and HLA-DR were also expressed by inflammatory cells as well as by endothelial cells in both the ACD and the ICD reactions.

DISCUSSION

The aim of the present study was to investigate whether there are differences between ACD and ICD reactions in the expression of the CXC chemokines IP-10, Mig, and IP-9. These CXC chemokines specifically interact with the receptor CXCR3 which is nearly exclusively expressed on activated T cells. Radioactive mRNA *in situ* hybridization was used to detect chemokine expression at different time-points of well-defined experimentally induced ACD reactions and SLS-induced ICD reactions.

Our results clearly demonstrate differences in the expression of IP-10, Mig, and IP-9 mRNA between ACD and ICD reactions. In ACD reactions, a time-dependent increase of chemokine mRNA expression was observed which concurred with the influx of T cells. At 24 h the amount of chemokine expression is still very low and is preceded by small amounts of T cells at the dermal–epidermal junction. This suggests that the initial recruitment of these cells is controlled by other chemotactic factors. At later time-points (48 and 72 h) the increased expression of IP-10, Mig, and IP-9 indicates involvement of these chemokines in the amplification of the inflammatory process. IP-10 was most abundant, and predominantly expressed by epidermal cells. Mig was expressed in both epidermis and dermis, whereas IP-9 was mainly expressed by epidermal cells, although to a much lower extent compared with IP-10. In contrast, the ICD reactions were consistently negative. In addition to chemokine expression, up to 50% of the infiltrating cells in the ACD reactions expressed CXCR3, the cognate receptor for IP-10, Mig, and IP-9, whereas at most 30% of the cells in the ICD reaction expressed CXCR3. Recent double staining experiments on skin biopsies from various inflammatory dermatoses, including some of the ACD and ICD reactions presented herein, indicate that both in ACD and ICD reactions CXCR3 is expressed by CD4 + as well as CD8 + T cells (Flier *et al*, unpublished observations).

The differential expression of IP-10 in human ACD and ICD reactions is consistent with the results of previous studies in mice (Enk and Katz, 1992) and suggests that this CXCR3 activating chemokine plays an important part in the generation of the inflammatory infiltrates in ACD in humans, but not in the SLS-induced ICD reactions. Furthermore, we show here that the related CXC chemokines Mig and IP-9 are also exclusively expressed in ACD reactions. Expression of IP-10 and Mig has also been found in other skin diseases like cutaneous T cell lymphomas (Tensen *et al*, 1998), psoriasis (Goebeler *et al*, 1998), and lichen planus (Spandau *et al*, 1998).

In addition to the differences in chemokine mRNA and chemokine receptor expression, we observed other, less pronounced differences between the ACD and ICD reactions. First, the dermal infiltrates in ACD were more pronounced than in ICD, even if the clinical score was identical. Similar observations have been made by other groups (Scheynius *et al*, 1984; Gawkrodger *et al*, 1987). Second, whereas neutrophils were rare in ACD, considerable numbers of neutrophils were found in SLS-induced ICD reactions. They were most pronounced in cases with focal necrosis and peaked at 24 h. Consistently, we found interleukin-8 mRNA in SLS-induced ICD reactions using *in situ* hybridization (Flier *et al* unpublished observations). Interleukin-8 attracts neutrophils by

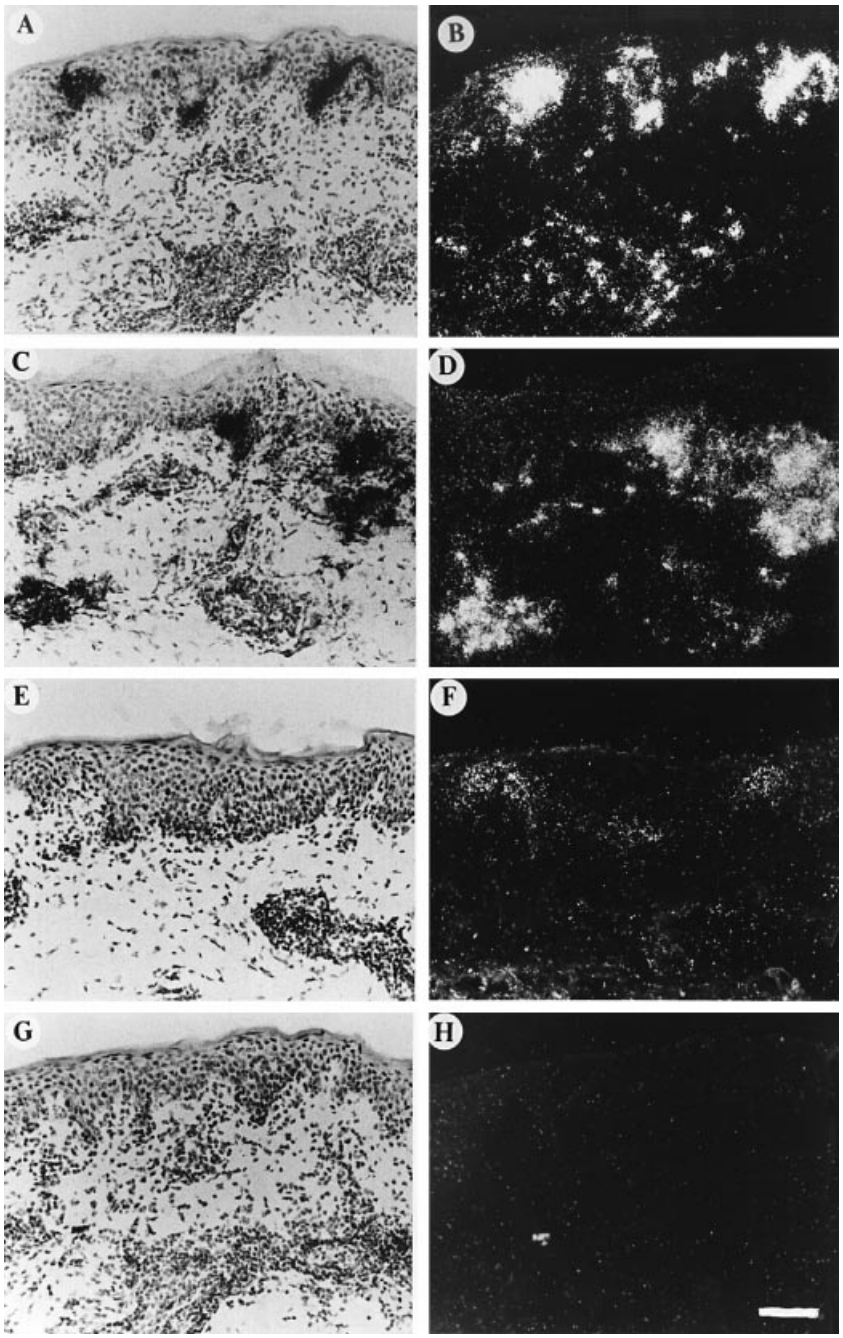

Figure 1. Characteristic patterns of IP-10, Mig, and IP-9 mRNA expression in an allergic contact dermatitis reaction. *In situ* hybridization with ^{35}S-UTP-labeled anti-sense RNA and sense RNA probes followed by emulsion autoradiography was performed on cryostat sections of an ACD reaction as described in *Materials and Methods*. Silver grains representing sites of IP-10 mRNA transcripts are found in both the epidermis and dermis: (*A*) bright-field and (*B*) dark-field illumination of the same section. Mig mRNA was detected more pronounced in the dermis compared with the epidermis; (*C*) bright-field and (*D*) dark-field illumination. IP-9 mRNA was focally expressed mainly by epidermal cells: (*E*) bright-field and (*F*) dark-field illumination. Specificity of the hybridization procedure was demonstrated using an IP-10 sense RNA probe on a serial section of the same biopsy showing absence of silver grains: (*G*) bright-field and (*H*) dark-field illumination. *Scale bar:* (*a–h*) 140 mm.

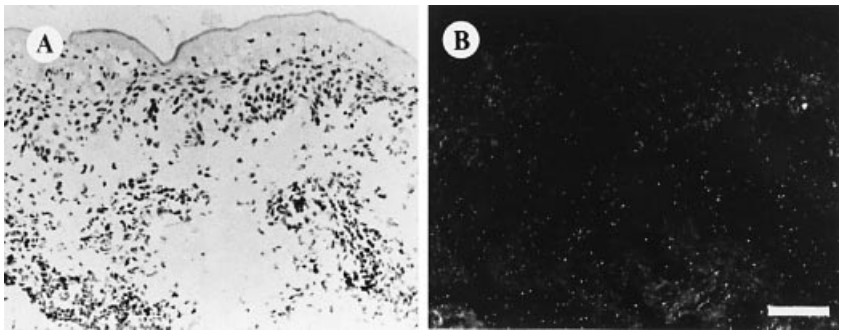

Figure 2. Absence of IP-10 mRNA expression in a representative section of a SLS-induced ICD reaction. *In situ* hybridization with ^{35}S-UTP-labeled IP-10 anti-sense RNA followed by emulsion autoradiography was performed on cryostat sections as described in *Materials and Methods*. Absence of silver grains reflects lack of expression of IP-10 mRNA in the SLS-induced ICD reaction; (*A*) bright-field and (*B*) dark-field illumination of the same section. *Scale bar:* (*a,b*) 140 mm.

binding to either the CXCR1 or CXCR2 expressed on neutrophils (Hébert and Baker, 1993; Chuntharapai *et al*, 1994).

Furthermore, immunohistochemical evaluation of ICAM-1 and HLA-DR expression by keratinocytes revealed expression of ICAM-1 after 24 h and HLA-DR after 72 h in ACD reactions only. Previous studies on ICAM-1 and HLA-DR expression have revealed some conflicting data, probably due to differences in allergens and irritants used and in the time of biopsy. ICAM-1 and HLA-DR expression have been reported to be expressed in ACD (Lange Vejlsgaard *et al*, 1989; Garioch *et al*, 1991) and also in ICD

(Gawkrodger *et al*, 1987; Willis *et al*, 1991; Brasch *et al*, 1992). For HLA-DR expression, 72 h might have been too early according to *in vivo* observations done by Scheynius and Fischer (1986).

The results of our study, showing explicit differences in expression of IP-10, Mig, and IP-9 as well as more gradual differences in a number of histologic and immunohistochemical parameters, seem to reflect the different pathogenic mechanisms involved in the development of ACD and ICD reactions.

As IFN-γ induces not only the expression of ICAM-1 and HLA-DR but also the expression of CXCR3 activating CXC chemokines, it is reasonable to assume that correlation between the expression of IP-10, Mig, IP-9, and ICAM-1 can be explained by the local presence of IFN-γ in these ACD reactions. Recently, in allergic individuals, activated T cells producing IFN-γ were identified (Cavani *et al*, 1998). Our data suggest that IFN-γ produced by these activated T cells might play a part in these ACD reactions by inducing the expression of IP-10, Mig, and IP-9. Epidermal keratinocytes are well known producers of these chemokines (Boorsma *et al*, 1998; Tensen *et al*, 1999a), although the identity of the producing cells in the dermis is difficult to establish. *In vitro* studies showed that both monocytes/macrophages, fibroblasts, and endothelial cells express the mRNAs encoding these chemokines upon stimulation with IFN-γ (Tensen *et al*, 1999b). As close examination of the sections revealed that endothelial cells were negative, both monocytes/macrophages and fibroblasts are possible sources of dermal chemokine mRNA in these ACD reactions. It appears that expression of these CXC chemokines by dermal and epidermal cells contributes to an environment in which activated T cells, bearing the CXCR3 receptor are able to migrate to the site of allergen reaction, thereby enhancing and maintaining the inflammatory infiltrate. In addition, the interaction between T cells and keratinocytes contributing to the production of chemokines and upregulation of adhesion molecules, appears to be crucial in the ACD reaction. Expression of IP-10, Mig, and IP-9 was not observed in SLS-induced ICD reactions, at least not in the first 72 h. Moreover, the interaction of the dermal infiltrates with the epidermis in these reactions was less pronounced. Yet, it remains unclear, which chemokines regulate the migration of inflammatory cells in an irritant reaction and thereby contribute to the development of the inflammatory response. With the mild damage to keratinocytes, irritants induce a cascade of common inflammatory mechanisms in which interleukin-1 and tumor necrosis factor-α are involved (Berardesca, 1997). Further research is required to elucidate the role of chemokines in ICD reactions not only induced by SLS but also by other irritants. If IP-10, Mig, and IP-9 are neither expressed in ICD reactions induced by other irritants, expression of these chemokines may be an important clue for differentiation between allergic and irritant contact reactions.

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